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Serum miR-133 as a novel biomarker for predicting treatment response and survival in acute myeloid leukemia

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Abstract. – OBJECTIVE: MiRNA-133 (miR-133) has been identified as a tumor suppressor in many types of human cancers. However, its clinical significance in acute myeloid leukemia (AML) is still unclear. The purpose of this study was to assess the correlation of miR-133 expression with clinical variables and prognosis in AML patients.

PATIENTS AND METHODS: Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) was performed to analyze blood samples from 145 patients with AML and 70 healthy volunteers.

RESULTS: Decreased miR-133 levels were observed in AML patients and closely associated with aggressive clinical parameters, such as white blood cells and poor Karyotype subgroups. In addition, receiver operator characteristic (ROC) analysis revealed that serum miR-133 could efficiently screen AML patients from normal controls with high sensitivity and specificity. More interestingly, serum miR-133 levels were remarkably elevated in the patients with favorable response after standard induction chemotherapy or achieving a complete remission. Furthermore, patients in the high serum miR-133 expression group had better overall survival and recurrence-free survival than those in the low serum miR-133 expression group. Meanwhile, multivariate analysis identified serum miR-133 as a significant independent predictor for survival.

CONCLUSIONS: Low miR-133 expression was a common event and correlated with worse clinical outcome in AML, suggesting that serum miR-133 might serve as a promising indicator for the early detection and prognosis evaluation of AML.

Key Words:
MiR-133, Acute myeloid leukemia, Biomarker, Survival.

Introduction

Acute myeloid leukemia (AML) is a complex neoplastic disease of the hematopoietic system and the most common acute leukemia in adults[1,2]. Although great advances have been achieved in the treatment of this malignancy over the past few years, the long-term survival rate of AML patients remains dismal mainly due to the high relapse rates[3,4]. Therefore, the identification of novel and reliable biomarkers with clinical importance is urgently needed.

Nowadays, a new class of short, noncoding RNAs named microRNAs (miRNAs), has been demonstrated to play an important role in neoplastic transformation[5,6]. It is well known that miRNAs play crucial roles in diverse cellular biological processes, including cell proliferation, differentiation, growth, migration and apoptosis. MiRNAs can act as either oncogenes or tumor suppressors, depending on their specific gene targets[7-9]. Increasing evidence has shown that miRNAs are dysregulated in many cancers, including AML. For instance, Han et al[10] reported that the expression level of miR-4262 in bone marrow and serum was markedly elevated compared to healthy controls, and miR-4262 overexpression in AML patients might predict an unfavorable prognosis. MiR-155 upregulation was associated with aggressive clinical variables and shorter overall survival of pediatric AML cases[11]. Conversely, miR-204 underexpression was a common event in AML patients and had an adverse impact on prognosis[12]. Despite this progress, more studies should be carried out to explore the functional role of miRNAs in AML.

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The miR-133 family has two members (miR-133a, miR-133b) which are different only at the last nucleotide of the 3' terminus. This miRNA was first experimentally characterized in mice and expressed in muscle tissue[13,14]. Yamamoto et al[15] showed that high EVI1 expression predicted poor prognosis of AML subjects, and miR-133 had anti-tumorigenic potential *via* inversely regulating EVI1 expression. However, the clinical significance of the serum miR-133 expression in AML was still lacking. In the present work, we investigated miR-133 level in serum from 145 AML cases and 70 healthy volunteers, and further demonstrated that the miR-133 expression was decreased and conferred a poor prognosis in patients with AML.

Patients and Methods

Patients and Blood Samples

Our study was approved by the Ethics Committee of The Second Hospital of Shanxi Medical University and the informed consent was collected from each participant. Blood samples were withdrawn from 145 *de novo* AML patients in this study. AML subjects with any other type of malignancy were excluded, and none of the cases underwent any chemotherapy or radiotherapy before sample collection. Moreover, blood samples of 70 healthy volunteers, 35 men and 35 women, 27 to 52 years of age, served as controls. Patient characteristics with respect to gender, age, white blood cells, platelets, bone marrow blasts, French-American-British subtypes and karyotype classification were described in Table I. All cases received standard induction chemotherapy consisting of 1 or 2 courses of daunorubicin (45 mg/m^2) for the last 3 days combined with cytarabine (100 mg/m^2) by a 7-day continuous intravenous infusion[16]. AML complete remission (CR) was defined as a normocellular BM containing less than 5% blasts and normalization of the peripheral blood counts after starting induction therapy[17]. Once CR had been achieved, patients received an entire course of consolidation chemotherapy. Approximately 7 ml of blood was collected from each participant in sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and immediately subjected to the three-spin protocol (1500 r.p.m. for 30 min, 3000 r.p.m. for 5 min, and 4500 r.p.m. for 5 min) to prevent contamination by cellular nucleic acids. Then, the supernatant was transferred to RNase/DNase-free tubes and stored at -80°C until further analysis.

RNA Isolation and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using a QIAamp RNA Blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The RNA sample concentration was quantified by NanoDrop ND-1000 (Nanodrop, Wilmington, DE, USA). Reverse transcription was performed to synthesize cDNA using MiScript Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed in triplicate on ABI 7500 fast Real Time-PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex

Table I. Clinical characteristics associated with miR-133 expression in patients with AML.

Characteristics	Low miR-133 (n = 68)	High miR-133 (n = 77)	*p*-value
Gender, male/female	32/36	45/32	0.170
Median age, years (range)	48 (21-78)	51 (20-75)	0.531
Median WBC, $\times 10^9$/L (range)	19.4 (0.64-254.8)	8.3 (0.51-369.8)	0.024
Median platelets, $\times 10^9$/L (range)	32.7 (6-426)	41 (7-485)	0.065
Median bone marrow blasts, % (range)	47.2 (14-96)	48.6 (17-98)	0.360
FAB subtypes			0.112
M0	33	25	
M1/M2	26	42	
M4/M5	9	10	
Karyotype classification			0.018
Favorable	14	4	
Intermediate	38	49	
Poor	16	24	

WBC, white blood cells; FAB, French-American-British.

Taq TM II (TaKaRa, Otsu, Shiga, Japan). The quantitative PCR values of all samples were normalized against the expression of cel-miR-39, and the relative expression of miR-133 was calculated by the comparative $2^{-\Delta\Delta Ct}$ method. The primer sequence of miR-133 is as follows: miR-133 Forward: 5′-TTTGGTCCCCTTCAACC-3′; miR-133 Reverse, 5′-GAGCAGGGTCCGAGGT-3′.

Statistical Analysis

The Mann-Whitney U test or Kruskal-Wallis test was performed to determine the significance of serum miR-133 levels among groups. The Chi-square test was used to evaluate the correlations between the results of serum miR-133 levels and various clinical features. Receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) were carried out to assess the feasibility of using serum miR-133 as a diagnostic tool for detecting AML. Kaplan-Meier method was used to plot overall survival (OS) and recurrence-free survival (RFS) curves. The difference in survival rates between the groups was performed with the log-rank test. OS was defined as the time between the diagnosis and death or the last follow-up. RFS was defined as the time between the diagnosis and induction failure, recurrence and death from any cause. Multivariate Cox proportional hazard models were employed to determine the associations between serum miR-133 levels and OS and RFS. All statistical analyses were performed using GraphPad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA, USA) and $p<0.05$ was considered to indicate a statistically significant result.

Results

Expression Level of MiR-133 in AML Patients and Its Diagnostic Accuracy

The serum miR-133 expression level in blood samples from all participants was detected with qRT-PCR. The data revealed that the relative miR-133 levels were greatly lower in AML subjects than those of healthy controls (Figure 1a, $p<0.05$). Next, the level of miR-133 in patients with the favorable Karyotype subgroup was significantly higher than in patients with intermediate/poor Karyotype subgroups, and serum miR-133 levels differed significantly between patients with intermediate Karyotype subgroup and poor Karyotype subgroup (Figure 1b, $p<0.05$). Then, a ROC curve analysis was used to illustrate the use of serum miR-133 in the differential diagnosis of AML. The analysis showed that serum miR-133 could serve as a valuable biomarker for identifying AML subjects from controls with an AUC of 0.839. The sensitivity and specificity of miR-133 were detected to be 83.4% and 75.7%, respectively (Figure 2).

Correlation Between Serum MiR-133 Expression and Clinical Factors

The relationship between serum miR-133 expression and clinicopathological variables were summarized in Table I. Using median expression as the cutoff, patients were divided into high and low expression groups. High miR-133 expression was significantly more frequently observed in patients with favorable Karyotype subgroup than other Karyotype subgroups ($p=0.018$). In addition, we noted a significant association between serum miR-133 expression and white blood cells

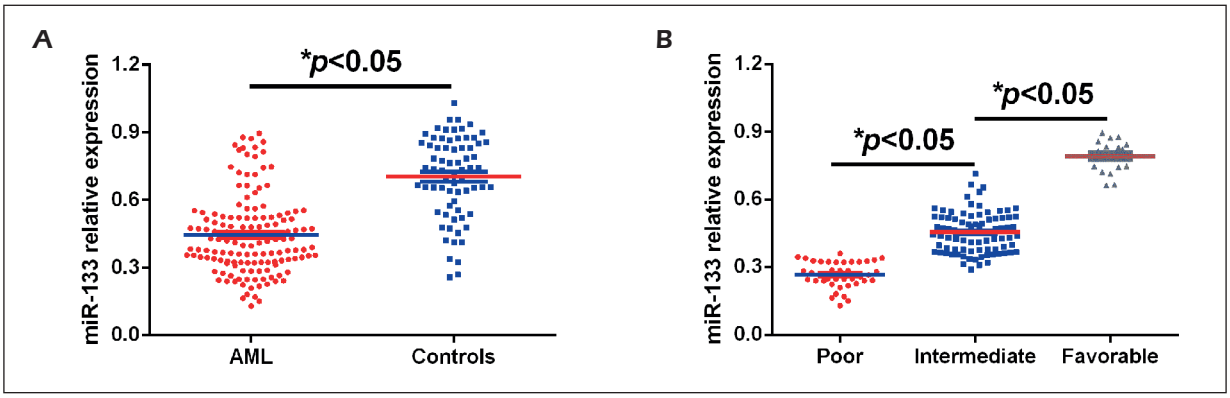

Figure 1. A, The serum miR-133 expression was significantly lower in AML patients compared with healthy individuals. **B,** The serum miR-133 expression was significantly lower in AML patients with poor Karyotype subgroup than that with favorable/intermediate Karyotype subgroups.

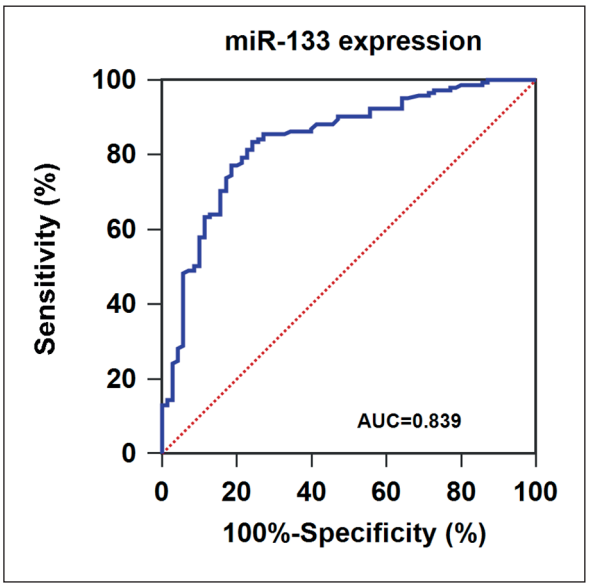

Figure 2. Diagnostic performance of serum miR-133 for AML.

(p=0.024). However, statistical analysis showed no significant correlation between miR-133 expression and gender, age, platelets, bone marrow blasts and FAB subtypes (all p>0.05).

Serum MiR-133 Expression Was Increased After Treatment

After the standard induction chemotherapy, 57 subjects were observed to be with a favorable response. We then collected their matched-pair blood samples, measured the miR-133 level with qRT-PCR and found that the levels of miR-133 in these cases were significantly upregulated (Figure 3a, p<0.05). We also collected the post-operative blood samples of 45 subjects with the achievement of CR for comparison; the results revealed that, in all CR patients, miR-133 levels were markedly increased (Figure 3b, p<0.05). The data suggested that miR-133 expression was sensitive to treatment response and could be a biomarker for clinical outcome.

Prognostic Impact of MiR-133 Expression

We further assessed the association between serum miR-133 expression and survival of AML patients, and complete follow-up data were available for all the cases. We found decreased miR-133 expression was strongly correlated with adverse survival of patients. Patients in the high miR-133 expression group had a longer OS (p=0.0045, Figure 4a) and RFS (p=0.0012, Figure 4b) than those in the low expression group. Next, we performed a multivariate Cox analysis to evaluate the influence of serum miR-133 levels and clinical characteristics (white blood cells, Karyotype classification) on patient survival. The results of the multivariate analysis indicated that serum miR-133 expression (OS: RR=3.46, 95% CI=1.17-5.92, p=0.013; RFS: RR=4.06, 95% CI=1.45-6.79, p=0.009) was an independent prognostic factor besides white blood cells and Karyotype classification (Table II).

Discussion

AML, the most common type of acute leukemia occurring in adults, is a heterogeneous malignancy characterized by differentiation arrest and malignant proliferation of clonal myeloid precursors[18]. It was previously reported that miR-

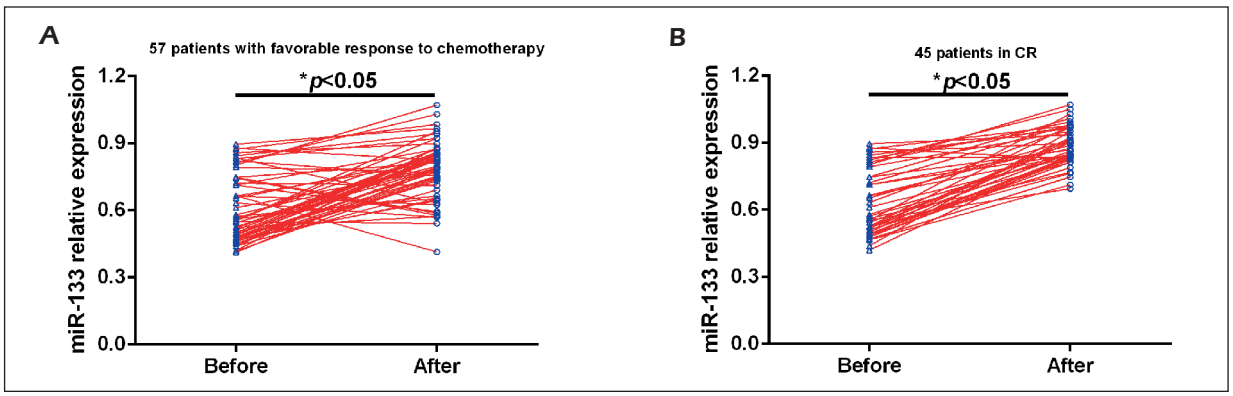

Figure 3. A, Changes in serum miR-133 levels in 57 patients with favorable response after standard induction chemotherapy. **B,** Changes in serum miR-133 levels in 45 patients achieving a CR.

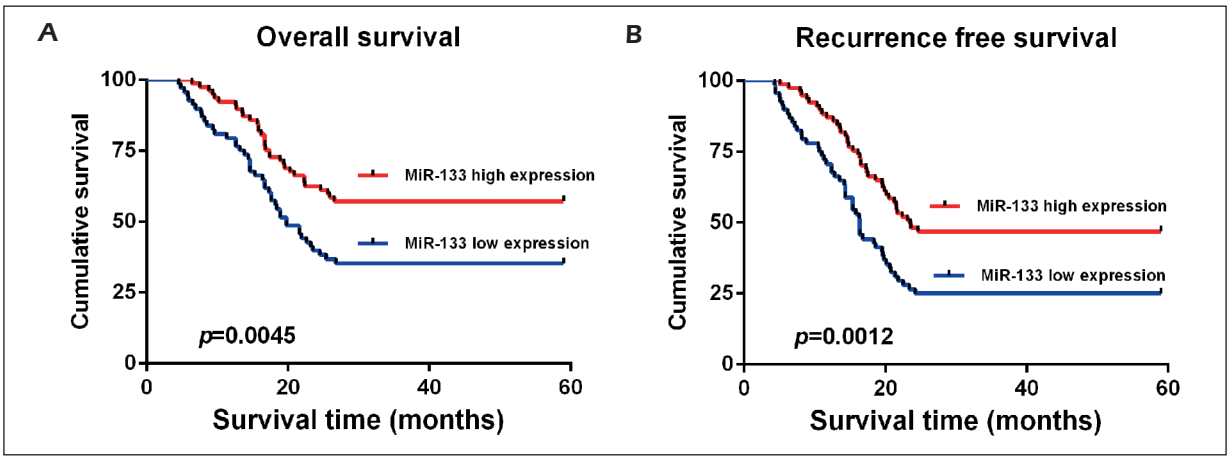

Figure 4. A, AML patients in the low miR-133 expression group had shorter overall survival than those in the high expression group. **B,** AML patients in the low miR-133 expression group had shorter recurrence free survival than those in the high expression group.

133 had a tumor-suppressive activity in AML[15]; the current work also showed that serum miR-133 downregulation was closely correlated with aggressive clinical parameters. MiR-133 levels were significantly reduced in AML patients compared with healthy controls, and there was a significant difference in serum miR-133 level between favorable Karyotype subgroup and intermediate/poor Karyotype subgroups. Moreover, the ROC curve revealed that serum miR-133 was able to distinguish AML from normal controls with AUC of 0.839. Additionally, a significant increase in serum miR-133 levels was observed when patients with the favorable response after standard induction chemotherapy or achieving a CR. Also, patients with low miR-133 expression were likely to be correlated with shorter OS and RFS. In addition, multivariate Cox regression analysis revealed that serum miR-133 expression was an independent predicting factor for AML. Our findings suggested miR-133 acted as a tumor suppressor in AML.

In addition to AML, miR-133 was widely reported to be dysregulated in various tumors. Interestingly, inverse correlations between some genes such as forkhead box C1 (FOXC1), epidermal growth factor receptor (EGFR) and miR-133 was previously studied. In glioma, Liu et al[19] revealed that miR-133 levels were significantly downregulated in cancerous tissues and cell lines. *In vitro* evidence showed that miR-133 upregulation markedly inhibited cell growth and invasion by suppressing FOXC1 levels. Likewise, a negative correlation between miR-133 and FOXC1 expression was observed in the pituitary tumor, and overexpression of miR-133 ex-

Table II. Multivariable analysis of the impact of variables on OS and RFS in 145 AML patients.

Variables	Risk Ratio	95% CI	p
Overall survival (all AML)			
White blood cells	2.43	0.72-4.22	0.025
Karyotype classification			
Intermediate *vs.* favorable	2.86	0.82-5.06	0.018
Poor *vs.* favorable	4.25	1.57-7.13	0.007
MiR-133 expression	3.46	1.17-5.92	0.013
Recurrence free survival (all AML)			
White blood cells	2.98	0.90-5.24	0.017
Karyotype classification			
Intermediate *vs.* favorable	3.23	1.02-5.67	0.014
Poor *vs.* favorable	4.72	1.74-7.95	0.003
MiR-133 expression	4.06	1.45-6.79	0.009

pression remarkably suppressed carcinogenesis of pituitary tumor malignancy[20]. In glioblastoma multiforme (GBM), low miR-133 expression occurred more frequently in GBM tissues compared with paired normal tissues. Depletion of miR-133 remarkably enhanced tumorigenesis of GBM through the regulation of EGFR and *vice versa*[21]. Similarly, Zhou et al[22] showed that the overexpression of miR-133 not only reduced EGFR expression, but also inhibited bladder cancer cell proliferation, migration and invasion. Tao et al[23] also demonstrated elevated miR-133 expression significantly decreased cell proliferation, migration and invasion by targeting EGFR in prostate cancer. In addition, some genes were also reported to be negatively associated with miR-133 expression. Li et al[24] found that miR-133 expression was greatly decreased in both tissues and cell lines of pancreatic cancer, and inhibited cell growth, migration and invasion by negatively regulating MIAT. The down-regulation of miR-133 was also indicated in lung cancer by Xiao et al[25] in 2016, and restoration of miR-133 dramatically attenuated cancer cell migration and invasion by directly silencing FOXQ1 expression. In gastric cancer (GC), the overexpression of miR-133 repressed GC cell proliferation and migration *via* interaction with the CDC42/PAKs pathway. Furthermore, low miR-133 expression was strongly correlated with aggressive clinical parameters and identified as an independent prognosis factor for overall survival of GC[26]. These results reported enforced miR-133 expression could reduce tumor initiation and progression, and were consistent with our findings.

Conclusions

To the best of our knowledge, our study is the first to report the potential use of serum miR-133 in AML diagnosis. In this work, we found that serum miR-133 levels were reduced in AML cases and inversely associated with worse clinical features. More importantly, low serum miR-133 could distinguish AML from normal controls and predicted shorter patient survival. Based on these results, serum miR-133 might serve as a useful marker for the diagnosis and prognosis of AML.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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